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Kajimura et al. J. CELL BIOL. 95, page 217A (Nov. 1982)

Larhammer et al. PNAS 79 3687-3691 (June 1982)

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Owerbach et al. PNAS 80 3758-3761 (jUNE 1983).

THANKS

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Genetics

Detection of HLA-D/DR-related DNA polymorphism in HLA-D homozygous typing cells

(HLA-DR/gene polymorphism/Southern blot analysis)

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ABSTRACT Sequences of different sizes are generated when DNA from homozygous HLA-Dw/DR typing cells are digested with restriction endonuclease and analyzed by hybridization with a HLA-D region class II antigen β-chain cDNA probe. The patterns of hybridization were highly polymorphic but one endonuclease, *Bam*HI, defined sequences unique to all HLA-Dw/DR specificities 1–8 except HLA-Dw/DR 2 and 6; however, these two specificities were resolved with the enzyme *Eco*RI. Digestion with other endonucleases such as *Pst* I results in patterns of restriction fragments that differ between homozygous typing cells of the same HLA-Dw/DR specificity. HLA-D region β-chain probes permit HLA-D region genotyping at the DNA level and may allow detection of genes controlling the association of HLA specificities with a wide variety of diseases.

The HLA-D region class II antigens are membrane proteins that are expressed on many cells involved in the immune response. The antigens are highly polymorphic and seem to determine a number of cellular immune functions (1, 2). The HLA-Dw/DR determinants defined by serological and mixed lymphocyte culture test are associated not only with certain diseases, often of autoimmune character (3, 4), but also with characteristics of the immune response to antigens in normal individuals (5–7). Present evidence suggests that class II antigens are encoded by several loci (DR, DC, SB) on the short arm of chromosome 6 (cf. ref. 8). The polymorphic character of the class II antigens resides in the β chain, whereas the α chain appears less heterogeneous (9–11). Restriction enyzme polymorphism analysis of genomic DNA with cloned cDNA of class II antigen α-chain or β-chain mRNA showed single hybridization bands with α-chain (12–14) but showed multiple bands with β-chain probes (14, 15).

In the present study we have used the HLA-D region β-chain cDNA probe pDR-β-1 (11, 16) to test whether it will hybridize to different fragments of restriction endonuclease-digested DNA prepared from established homozygous typing cells representing the HLA-Dw/DR specificities 1–8 and from cells with specificities defined as SN and DH (17).

MATERIALS AND METHODS

Homozygous Typing Cells. The lymphocytes used to define HLA-Dw types in the mixed lymphocyte reaction have been described in detail elsewhere (17): Lou, Mette, and Maja detect HLA-Dw1; MST, Ruth, and BA, HLA-Dw2; LK, LN, and LB, HLA-Dw3; BB and Leif, HLA-Dw4; Raff, HLA-Dw5; Arent and GP, HLA-Dw6; SL, GS, and GM, HLA-Dw7; and Mad, HLA-Dw8. All of these cells are also homozygous for the corresponding DR antigens, except for DR6 because monospecific DR6 antisera are not yet available (17). In addition, lymphocytes from two donors defining the specificities DH (17) and SN (18) were also tested. All cells were stored in liquid nitrogen and have been used in routine HLA-D typing for many years.

DNA Digestion and Binding to Filters. DNA (≈50 μg) was isolated from 5×10^6 homozygous typing cells by previously described methods (19). DNA samples (≈5 μg) were digested for 15–20 hr in a final volume of 100 μl with 27 units of *Eco*RI, 24 units of *Bam*HI, or 12 units of *Pst* I according to the manufacturer's (Boehringer Mannheim) specifications. The resulting DNA fragments were separated by gel electrophoresis on 1% agarose slab gels and transferred (20) to nitrocellulose filters (Schleicher & Schuell). Fragments of *Hin*dIII-digested λ DNA (Bethesda Research Laboratories) were used as molecular size markers.

Hybridization Probe. The probe for hybridization was derived from the recombinant plasmid pDR-β-1, which contains DNA sequences complementary to a class II antigen β-chain mRNA (11, 16), the sequence of which demonstrates homology to the mouse I-A class II antigens and is tentatively thought to represent a HLA-DC β chain (11). The pDR-β-1 cDNA was digested with *Pst* I and *Pvu* II and two fragments representing nucleotides 1–790 of the clone (14) were isolated by agarose gel electrophoresis, followed by electroelution into dialysis tubing. These restriction fragments correspond to most of the coding region and some 3′ nontranslated sequences and do not contain naturally occurring *Eco*RI, *Bam*HI, or *Pst* I restriction sites (11, 16). The fragments were labeled with [^{32}P]dCTP (New England Nuclear) to specific activities of ≈10^8 cpm/μg by nick-translation (21).

Hybridization and Washing of Filters. Before hybridization, nitrocellulose filters were first treated for 7–12 hr at 42°C in 50% formamide containing 0.75 M NaCl, 75 mM trisodium citrate, 50 mM sodium phosphate buffer (pH 6.5), 500 μg of sonicated denatured herring sperm DNA (Pel-Freez Biochemicals) per ml, and 5× concentrated Denhardt's reagent (22). Filters were then placed in 50% formamide/0.75 M NaCl/75 mM trisodium citrate/20 mM sodium phosphate buffer, pH 6.5/200 μg of sonicated denatured herring sperm DNA per ml/Denhardt's reagent in addition to 10% (wt/vol) sodium dextran sulfate 500 (Pharmacia) (23) and ^{32}P-labeled heat-denatured probe (5×10^5 cpm/ml). After 15–24 hr at 42°C filters were washed three times at room temperature with 0.3 M NaCl/30 mM trisodium citrate/0.1% $NaDodSO_4$ for 10 min each, followed by three additional washes at 50°C for 20 min each with 15 mM NaCl/1.5 mM trisodium citrate/0.1% $NaDodSO_4$. Labeled DNA se-

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Abbreviation: kb, kilobase(s).

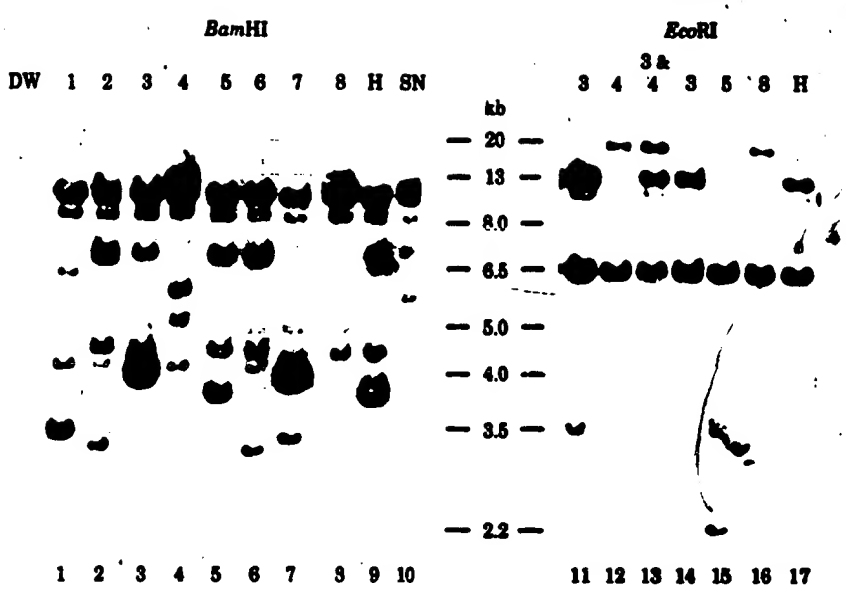

FIG. 1. Autoradiogram of blot hybridization analysis of homozygous HLA-Dw typing cells with the HLA-DR β probe. Lanes 1–10 were digested with *Bam*HI and lanes 11–17, with *Eco*RI. The individual lanes were DNA from: lane 1, Mette; lane 2, Ruth; lane 3, LK; lane 4, BB; lane 5, Raff; lane 6, GP; lane 7, GM; lane 8, Mad; lane 9, Herluf; lane 10, SN; lane 11, LB; lane 12, Leif; lane 13, mixture of LN and BB cells; lane 14, LK; lane 15, Raff; lane 16, Mad; and lane 17, Herluf.

quences were detected by exposing the filters to x-ray film (XAR-5, Kodak) for 3–7 days at −70°C in the presence of an intensifying screen (DuPont).

RESULTS

***Eco*RI and *Bam*HI Restriction in HLA-Dw Homozygous Typing Cells.** DNA isolated from each homozygous typing cell was digested with *Eco*RI and *Bam*HI. The β-chain cDNA probe hybridized to 10 *Eco*RI-cleaved DNA fragments of approximately 2.2, 3.5, 3.8, 3.9, 4.0, 6.5, 9.0, 11.0, 13.0, and 20.0 kilobases (kb) (Fig. 1 *Right*) and to 12 *Bam*HI (Fig. 1 *Left*) sequences of 3.2, 3.4, 3.7, 4.0, 4.3, 5.0, 5.8, 6.2, 7.0, 8.0, 10.0, and 12.0 kb. Only the 11.0- and 6.5-kb *Eco*RI sequences and 10.0-, 8.0-, and 5.0-kb *Bam*HI sequences were present in all typing cells. All other sequences were variable, being present in some but not all typing cells. These sequences are summarized in Table 1 for *Bam*HI and in Table 2 for *Eco*RI. For example, Mette (Fig. 1, lane 1) had *Bam*HI sequences of 6.2 and 3.4 kb, which were absent in Ruth (Fig. 1, lane 2), whereas Ruth had *Bam*HI sequences of 7.0, 4.3, and 3.2 kb that were absent in Mette. Likewise, with *Eco*RI-cleaved DNA, Mette had a sequence of 13.0 kb that was absent in Ruth, whereas Ruth had 9.0- and 2.2-kb sequences that were absent in Mette (Table 2). The specific *Eco*RI and *Bam*HI restriction sequences thus produced a distinct pattern for different individuals. Eight and seven different band patterns were detected by using *Bam*HI (Table 1) and *Eco*RI (Table 2), respectively. The *Bam*HI digest revealed that all Dw/DR specificities were different, except between Dw/DR 2 and 6 as well as between Dw/DR4 and SN (Table 1). The *Eco*RI digest revealed identical fragments only in the Dw/DR4, 7, and SN cells as well as in Dw/DR3 and DH (Table 2). However, all HLA-Dw/DR specificities could be resolved in the combined *Eco*RI and *Bam*HI analysis, with the exception of SN. In addition, neither *Eco*RI nor *Bam*HI could resolve the different homozygous typing cells of the same HLA-Dw/DR type—i.e., Lou, Mette, and Maja all had the same restriction map.

HLA-D/DR-like DNA Polymorphism With Other Restriction Endonucleases. Because neither *Eco*RI nor *Bam*HI could resolve homozygous typing cells of the same HLA-Dw/DR type, it was of interest to determine if other restriction endonucleases could further resolve common HLA-Dw/DR types into different restriction maps. DNA from homozygous typing cells

Table 1. *Bam*HI restriction sequences in homozygous typing cells

Typing cell	Homozygous for Dw	*Bam*HI sequences, kb								
		12	7.0	6.2	5.8	4.3	4.0	3.7	3.4	3.2
Lou, Mette, Maja	1			+			+		+	
MST, Ruth, BA, Arent, GP	2, 6		+			+	+			+
LK, LN, LB	3		+			+	+*			
BB, Leif, SN	4, SN	+	+†		+		+			
Raff	5		+			+		+	—	+
SL, GS, GM	7		+†			+	+*		+	
Mad	8	+				+				
Herluf	H		+			+		+		

Sequences of 10.0, 8.0, and 5.0 kb were present in all cells and are not listed.
* Strong hybridization signal.
† Weak hybridization signal.

Table 2. *Eco*RI restriction sequences in homozygous typing cells

Typing cell	Homozygous for HLA-Dw	*Eco*RI sequence, kb					
		20	13	9.0 and 2.2	4.0 and 3.8*	3.9	3.5
Lou, Mette, Maja	1		+			+	+*
MST, Ruth, BA	2			+		+	+
LK, LN, LB, Herluf	3, H		+				+
BB, Leif, SL, GS, GM, SN	4, 7, SN	+			+		
Raff	5	+		+			+
Arent, GP	6			+			+
Mad	8	+					+

Sequences of 11.0 and 6.5 kb were present in all cells and are not listed.
*Weak hybridization signal.

Arent, GP, BB, Leif, and SN were digested with the restriction endonucleases *Pst* I, *Bgl* I, *Pvu* II, *Hin*fI, or *Hin*dIII, respectively. The restriction sequences generated from the *Pst* I digest revealed the greatest degree of polymorphism (data not shown). Subsequently, DNA from the remaining homozygous typing cells were digested with *Pst* I (Fig. 2). Although the homozygous typing cells of identical HLA-D types could not be resolved with *Eco*RI or *Bam*HI, many were resolved with *Pst* I (Fig. 2 and Table 3). For example, LB had an 18-kb sequence that was absent in LK and LN; all of these three cells were homozygous for Dw3 (Fig. 2). In addition, *Pst* I digestion resolved BB, Leif, and SN into different restriction maps (Fig. 2 and Table 3). Only in the three HLA-Dw/DR-2 cells was additional resolution not obtained (Table 3).

DISCUSSION

The use of the pDR-β-1 cDNA (11, 16) to probe restriction enzyme-digested DNA from homozygous typing cells provides evidence that the fragment polymorphism is associated with HLA-DR genotypes, as determined by serological methods. However, the present β-chain cDNA probe was isolated from a cDNA clone separate from the HLA-DR β-chain locus (11, 16, 24). It does not correspond to the I-E/C region, the murine counterpart to human HLA-DR, but rather to the human equivalent of the murine I-A β-chain locus (16, 24). Previous hybridization analyses with various β-chain probes (14) showed restriction enzyme patterns of human DNA that suggested the presence of at least three distinct β-chain genes or pseudogenes. This is in contrast to similar analyses with cDNA probes for the HLA-D region α chain, which have shown only a single component (12–14). It is notable that the complexity of restriction fragments detected with the β-chain probe is considerably smaller than with probes for class I transplantation antigens (25).

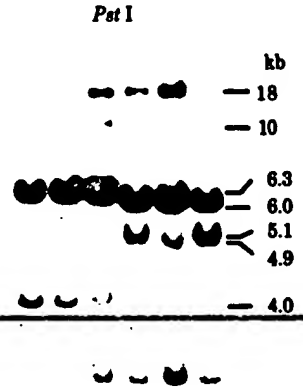

FIG. 2. Autoradiogram of blot hybridization analysis of homozygous HLA-Dw typing cell DNA digested with *Pst* I. Lane 1 (LK), lane 2 (LN), and lane 3 (LB) are HLA-Dw3; lanes 4 (BB) and 5 (Leif) are HLA-Dw4; and lane 6 (SN) is HLA-Dw SN.

Our homozygous tissue typing cells are used as stimulator cells in the mixed lymphocyte culture to define HLA-D determinants (17) and several of them have been included in various international histocompatibility workshops (8, 17), which also have proven their reliability as typing reagents. Therefore, it is remarkable that digestion with *Bam*HI resulted in patterns of restriction fragments that were unique to nearly each of the HLA-Dw/DR types defined by these cells, the only exceptions being Dw2 and Dw6. Digestion with *Eco*RI permits these two specificities to be distinguished. Thus, the *Bam*HI and *Eco*RI sequences detected are likely to be closely linked to the HLA-Dw/DR specificities. On the other hand, the results with *Pst* I suggest that our probe detects heterozygosity within the HLA-D region. Therefore, it is possible that the homozygous typing cells are not homozygous for all HLA-D β-chain loci or that restriction sites, located in pseudogenes, or intervening or flanking sequences are detected. The results obtained with the SN cell are particularly interesting because this cell is DR 4/4 homozygous, but Dw4-negative. However, in the mixed lymphocyte culture test it types for a determinant characteristic of most DR4-positive, Dw4-negative individuals. The *Bam*HI and *Eco*RI patterns group this cell together with the Dw/DR4 cells and, although the *Pst* I pattern of SN is unique, it has strong resemblance to the Dw4 cells. Therefore, it is possible that these similarities at the DNA level may reflect serological crossreactivity between SN and Dw4 at the protein level.

Our results demonstrate that the pDR-β-1 cDNA probe is able to define HLA-Dw/DR types normally detected by either serological or cell culture techniques. However, this cDNA probe appears to unveil additional polymorphism not detected by the latter methods. Further studies with the present and other β-chain probes (14, 15) may make it possible to identify subtypes of the serologically defined HLA-DR types and to test whether fragments generated with endonucleases are related to the well-known association of tissue types with susceptibility of diseases.

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Table 3. *Pst* I restriction sequences in homozygous typing cells

HLA-D specificity	Workshop designation*	Typing cells	HLA-ABC phenotype	Restriction fragment length, kb									
				18	10	7.2	6.5	6.3	6.0	5.4	5.1	4.9	4.0
Dw1		Lou	A3;Bw35;Cw4							+	+		
		Mette	A2,3;B5,18							+	+		
		Maja	A2,3;Bw35;Cw4	+						+	+		
Dw2	8w117	MST	A3;B7	+			+						
		Ruth	A2,3;B7	+			+						
		BA	A3;B7	+			+						
Dw3		LK	A1;B8		+			+					+
	8w125	LN	A1;B8		+			+					+
		LB	A1;B8	+	+			+					+
Dw4	8w135	Leif	A2;B15;Cw3	+					+			+	
		BB	A2;B15;Cw3	+					+		+	+	
Dw5		Raff†	A1;Bw35;Cw4	+					+		+		
Dw6	8w152	Arent	A2;Bw38,39	+	+						+		+
		GP	A3;Aw32;Bw38	+							+		
Dw7		SL	A2,28;B44			+		+			+		
	8w160	GM†	A29;B44			+		+			+		
		GS	A3,11;B7,44		+	+		+					+
SN		SN	A3;B27;Cw2						+		+	+	
DH	8w319	Heriuf	A2;Bw35,44;Cw4,5	+					+		+		
Dw8	8w204	Mad†	A2;B40;Cw3			NT							

A number of restriction sequences smaller than 4.0 kb were present in all cells and are not listed. NT, not tested.
*The Eighth Histocompatibility Workshop designation (8).
†First cousin offsprings.

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